

MBHB CASE NO. 99,274-C
RECOMBINANT LAMININ 5

Cross Reference

5 This application claims priority to U.S. Provisional Patent Application Serial Nos. 60/131,720 filed April 30, 1999; 60/149,738 filed August 19, 1999; and 60/155,945 filed September 24, 1999, all of which are incorporated herein by reference in their entirety.

10 **Field of the Invention**

This application relates to recombinant laminin 5 and methods for its use.

Background of the Invention

15 Basal laminae (basement membranes) are sheet-like, cell-associated extracellular matrices that play a central role in cell growth, tissue development, and tissue maintenance. They are present in virtually all tissues, and appear in the earliest stages of embryonic development.

20 Basal laminae are central to a variety of architectural and cell-interactive functions. (See for example, Malinda and Kleinman, Int. J. Biochem. Cell Biol. 28:957-959 (1996); Aumailley and Krieg, J. Invest. Dermatology 106:209-214 (1996)):

- 25 1. They serve as architectural supports for tissues, providing adhesive substrates for cells.
2. They create perm-selective barriers between tissue compartments that impede the migration of cells and passively regulate the exchange of macromolecules. These properties are illustrated by the kidney glomerular basement membrane, which functions as an important filtration structure, creating an effective blood-tissue barrier that is not permeable to most proteins and cells.
- 30 3. Basal laminae create highly interactive surfaces that can promote cell migration and cell elongation during embryogenesis and wound repair. Following an injury, they provide a surface upon which cells regenerate to restore normal tissue function.
4. Basal laminae present information encoded in their structure to contacting cells that is important for differentiation and tissue maintenance. This information is communicated

to the cells through various receptors that include the integrins, dystroglycan, and cell surface proteoglycans. Signaling is dependent not only on the presence of matrix ligands and corresponding receptors that interact with sufficient affinities, but also on such topographical factors as ligand density in a three-dimensional matrix "landscape", and on the ability of basal lamina components to cluster receptors. Because these matrix proteins can be long-lived, basal laminae create a "surface memory" in the basal lamina for resident and transient cells.

The basal lamina is largely composed of laminin and type IV collagen heterotrimers that in turn become organized into complex polymeric structures. To date, six type IV collagen chains and at least twelve laminin chains (and twelve different heterotrimeric laminins) have been identified. These chains possess shared and unique functions and are expressed with specific temporal (developmental) and spatial (tissue-site specific) patterns.

Laminins are a family of heterotrimeric glycoproteins that reside primarily in the basal lamina. They function via binding interactions with neighboring cell receptors, and are important signaling molecules that can strongly influence cellular function. Laminins are important in both maintaining cell/tissue phenotype as well as promoting cell growth and differentiation in tissue repair and development.

Laminins are large, multi-domain proteins, with a common structural organization. The laminin molecule integrates various matrix and cell interactive functions into one molecule.

The laminin molecule is comprised of α -, β -, and γ -chains joined together through a coiled-coil domain. Within this structure are identifiable domains that possess binding activity towards other laminin and basal lamina molecules, and membrane-bound receptors. Domains VI, IVb, and IVa form globular structures, and domains V, IIIb, and IIIa (which contain cysteine-rich EGF-like elements) form rod-like structures. (Kamiguchi et al., Ann. Rev. Neurosci. 21:97-125 (1998)). Domains I and II of the three chains participate in the formation of a triple-stranded coiled-coil structure (the long arm).

Table 1 shows the individual chains that each laminin type is composed of:

TABLE 1. Known laminin family members

<i>Protein</i>	<i>Chains</i>
Laminin-1	$\alpha 1\beta 1\gamma 1$
Laminin-2	$\alpha 2\beta 1\gamma 1$
Laminin 3	$\alpha 1\beta 2\gamma 1$
Laminin-4	$\alpha 2\beta 2\gamma 1$
Laminin-5	$\alpha 3\beta 3\gamma 2$
Laminin-6	$\alpha 3\beta 1\gamma 1$
Laminin-7	$\alpha 3\beta 2\gamma 1$
Laminin-8	$\alpha 4\beta 1\gamma 1$
Laminin-9	$\alpha 4\beta 2\gamma 1$
Laminin-10	$\alpha 5\beta 1\gamma 1$
Laminin-11	$\alpha 5\beta 2\gamma 1$
Laminin-12	$\alpha 2\beta 1\gamma 3$

Four structurally-defined family groups of laminins have been identified. The first group of five identified laminin molecules all share the $\beta 1$ and $\gamma 1$ chains, and vary by their α -chain composition ($\alpha 1$ to $\alpha 5$ chain). The second group of five identified laminin molecules all share the $\beta 2$ and $\gamma 1$ chain, and again vary by their α -chain composition. The third group of identified laminin molecules has one identified member, laminin 5, with a chain composition of $\alpha 3\beta 3\gamma 2$. The fourth group of identified laminin molecules has one identified member, laminin 12, with the newly identified $\gamma 3$ chain ($\alpha 2\beta 1\gamma 3$)

Some progress has been made in elucidating the relationship between domain structure and function. (See, for example, Wewer and Engvall, Neuromusc. Disord. 6:409-418 (1996).) The overall sequence similarity among the homologous domains in different chains varies, but it is highest in domain VI (thought to play a key role in laminin polymerization), followed by domains V (possibly involved in protein-protein interactions) and III (entactin/nidogen binding; possible cell adhesion sites), and is lowest in domains I, II (both thought to be involved in intermolecular assembly, and containing possible cell adhesion sites), and G. Not all domains are present in all 3 types of chains. The globular G domain (thought to be involved in cell receptor binding) is present only in the α chains. Other domains may not be present in all chains within a certain chain type. For example, domain VI is absent from $\alpha 3$, $\alpha 4$, and $\gamma 2$ chains. (Wewer and Engvall, 1996)

As a result of their large size (>600 kD) and unique structure, the laminin molecules can be resolved in the electron microscope. (Wewer and Engvall, 1996) Typically, laminins appear as cross-shaped molecules in an EM. The three short arms of the cross represent the

amino terminal portions of each of the three separate laminin chains (one short arm per chain). The long arm of the cross is composed of the C-terminal parts of the three chains, which together form a coiled coil structure. (Wewer and Engvall, 1996) The long arm ends with the globular G domain.

5 The coiled-coil domain of the long arm is crucial for assembly of the three chains of laminin. (Yurchenco et al., Proc. Natl. Acad. Sci. 94:10189-10194 (1997)). Disulfide bonds bridge and stabilize all three chains in the most proximal region of the long arm and join the β and γ chains in the most distal region of the long arm.

10 A model of laminin receptor-facilitated self-assembly, based on studies conducted with cultured skeletal myotubes and Schwann cells, predicts that laminins bind to their receptors, which freely diffuse in a fluidic membrane, when ligand-free. Receptor engagement forces these receptors into a high local two-dimensional concentration, facilitating their mass-action driven assembly into ordered surface polymers. In this process, the engaged receptors are also reorganized, accompanied by cytoskeletal rearrangements. (Colognato, J. Cell Biol. 145:619-15 631 (1999)) This reorganization activates the receptors, causing signal transduction with the alteration of cell expression, shape and/or behavior.

15 One class of laminin receptors are the integrins, which are cell surface receptors that mediate many cell-matrix and cell-cell interactions. Integrins are heterodimers, consisting of an α and a β subunit. 16 α - and 8 β -subunits are known, and at least 22 combinations of α and β subunits have been identified to date. Some integrins have only one or a few known ligands, whereas others appear to be very promiscuous. Binding to integrins is generally of low affinity, and is dependent on divalent cations. Integrins, activated through binding to their ligands, transduce signals via kinase activation cascades, such as focal adhesion and mitogen-activated kinases. Several different integrins bind different laminin isoforms more or less specifically. (Aumailley et al., In The Laminins, Timpl and Ekblom, eds., Harwood Academic Publishers, Amsterdam. pp. 127-158 (1996))

20 Laminin-5, also referred to as kalinin, nicein, and epiligrin, plays a key role in modulating the behavior and activity of cells and tissues of epithelial origin, and is expected to have broad uses in clinical settings where increased epithelial attachment and hemidesmosome assembly are required. (Takeda et al., J. Invest. Dermatol. 1999 113(1):38-42) Laminin-5 is a principal adhesive ligand in the epidermal basal lamina, and has been shown to promote the

attachment of keratinocytes and epithelial cells to the basal lamina and underlying dermis, and also promotes hemidesmosome formation. (Burgeson et al. U.S. Patent No. 5,770,562; Quaranta and Hormia, U.S. Patent No. 5,422,264; Jones, U.S. Patent No. 5,541,106; Quaranta and Hormia, U.S. Patent No. 5,658,789; Hormia et al., J. Invest. Dermatol. 1995 Oct. 5 105(4):557-561).

Laminin 5 is also thought to be necessary for the healing of epithelial tissue wounds. (Goldfinger et al., J. Cell Sci. 1999; 112(Pt. 16):2615-2629) Pretreatment of human keratinocyte sheets for grafting with laminin 5 improves grafting efficiency. (Takeda et al., J. Invest. Dermatol. 1999 Jul; 113(1):38-42). The addition of laminin-5 accelerates the formation of a basement membrane in a skin equivalent model (Tsunenaga et al., *Matrix Biol.* 17(8-9):603-613, 1998). Laminin-5 also promotes epithelial cell attachment to a wide variety of biomaterials, including polymers, hydroxyapatite, and metals. (Jones et al., U.S. Patent No. 5,585,267; El Ghannam et al., J. Biomed. Mater. Res. 1998 Jul; 41(1):30-40)

Laminin 5 has further been demonstrated to promote the following:

15 1. Epithelial cell adhesion to the internal basal lamina of teeth (Mullen et al., J. Periodontal. Res. 1999 Jan 34(1):16-24; Hormia et al., J. Dent. Res. 1998 Jul; 77(7):1479-1485) and anchorage of ameloblasts (ie: enamel-producing cells) to the enamel matrix. (Yoshiba et al., Cell Tissue Res. 1998 Apr; 292(1):143-149)

20 2. Corneal epithelial cell adhesion. (Qin and Kurpaku, Exp. Eye Res. 1998 May 66(5):569-579)

3. Intestinal epithelial restitution. (Lotz et al., Am. J. Pathol. 1997 Feb;150(2):747-760)

25 4. In vitro expansion of epithelial cells by providing an efficient adhesion substrate for primary cell cultures, thus providing the basis for a wide range of new cell therapy applications. (Gonzales et al., Mol. Biol. Cell. 1999 Feb; 10(2):259-270; Baker et al., Exp. Cell Res. 1996 Nov 1; 228(2):262-270).

30 5. Proliferation of pancreatic beta islet cells (Todorov et al., Transplant. Proc. 1998 Mar; 30(2): 455; Quaranta and Jones, U.S. Patent No. 5,510,263; Halberstadt et al, U.S. Patent No. 5,681,587; Halberstadt et al., U.S. Patent No. 5,672,361), and T cells (Vivinus-Nebot et al., J. Cell Biol. 1999 Feb 8; 144(3):563-574)

Thus, laminin 5 has broad uses in clinical settings where increased epithelial attachment and hemidesmosome assembly are required. Laminin 5 can be used to promote wound healing and tissue regeneration. The application of exogenous laminin 5 has broad application for the accelerated healing of skin disorders, such as diabetic foot ulcers, venous ulcers, pressure sores, skin surgery, burns, and acute wounds. Exogenous laminin 5 may be used to directly treat a wound surface, or may be applied to a variety of medical devices and dermal grafts for skin, corneal, gastrointestinal, and periodontal epithelial wound healing. The use of laminin 5 provides enhanced biocompatibility of the device or graft, which leads to improved tissue integration and remodeling, reduced immune response, and reduced likelihood of infection.

5 Laminin 5 is also useful for the ex vivo and in vitro proliferation of various cell types, including but not limited to epithelial cells, pancreatic beta islet cells, and T cells, and tissue equivalents. Thus, a source of laminin 5 for tissue culture media or a media supplement, as well as cell growth substrates coated with laminin 5, would be particularly useful for the cultivation of these and other cell types.

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15 A good source and purification procedure for laminin-5 is needed to provide material for the development of the therapeutic and research applications mentioned above. Some cell lines secrete laminin-5, and procedures have been developed to purify laminin-5 from the processed cells and cell media. However, these methods are time consuming and capable of producing only small amounts of laminin 5. (Rouselle et al., J. Biol. Chem. 1995 270(23):13766-13770; Cheng et al., J. Biol. Chem. 1997, 272(50):31525-31532)

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A preferred method of production, however, is the use of recombinant DNA technology to engineer a cell line of choice to produce recombinant laminin-5. A recombinant-based method of laminin-5 production has several advantages over purification from tissue or isolation from cell lines in culture:

- 25 1. The recombinant produced protein is free of pathogens. While this is also true for endogenous cell culture produced protein, protein derived from human tissue carries a risk for contamination by HIV, hepatitis, and other infectious agents.
- 30 2. Expression levels of the protein, and hence yields, can be improved through the use of genetically engineered genes/vectors that enhance the production of the encoded protein.

3. It is possible to engineer additional peptide sequences to the protein chain that provides a binding site for a commercially viable affinity purification procedure.
4. The method can provide for the modification of protein structure/function through the addition, substitution, elimination, and/or other modifications of protein domain structures. For example, it may be desirable to introduce an integrin binding site (e.g. RGD), switch integrin recognition sites, or engineer in a stable binding site to a synthetic substrate. Thus, the creation of expression vectors that express laminin chains generates enormous flexibility for future uses and creates a basis for creating second generation "designer" laminins.

10 Previous studies have produced cells transfected with one or two of the laminin 5 chain-encoding DNA sequences, but none have produced recombinant heterotrimeric laminin 5, nor have they produced cell lines that secrete recombinant heterotrimeric laminin 5. (Gagnoux-Palacios et al., J. Biol. Chem. 271:18437-18444 (1996); Matsui et al., J. Biol. Chem. 270:23496-23503 (1995))

15 Thus, there exists a need in the art for recombinant heterotrimeric laminin 5 protein, methods for making recombinant laminin 5, and methods of using recombinant laminin 5 for wound healing and tissue regeneration, for use on a variety of medical devices and dermal grafts for skin, corneal, gastrointestinal, and periodontal epithelial wound healing, for the ex vivo and in vitro proliferation of various cell types, and for tissue culture media, media supplements, and
20 as a component of cell growth substrates.

Summary of the Invention

The present invention fulfills the need in the art for recombinant laminin 5 protein, methods for making recombinant laminin 5, and methods of using recombinant laminin 5 for the treatment of burns, for use on a variety of medical devices and dermal grafts for skin, corneal, gastrointestinal, and periodontal epithelial wound healing, for the ex vivo and in vitro proliferation of various cell types, and for tissue culture media, media supplements, and as a component of cell growth substrates.

30 In one aspect, the present invention provides cells that have been transfected with nucleic acid sequences encoding laminin α_3 , β_3 and γ_2 chains, wherein the cells express the individual

chains, which assemble into heterotrimeric recombinant laminin-5 (hereinafter referred to as "r-laminin 5"). r-laminin 5, or processed forms thereof, are secreted by the cell.

In another aspect, the present invention provides r-laminin 5, and methods for producing substantially purified r-laminin 5, or processed forms thereof.

5 In a further aspect, the present invention provides pharmaceutical compositions, comprising r-laminin 5, or processed forms thereof, together with a pharmaceutically acceptable carrier. Such pharmaceutical compositions can optionally be provided with other compounds with wound healing and tissue regeneration utility, such as other extracellular matrix components.

10 In further aspect, the present invention provides methods and kits for using r-laminin 5 to:

- a. accelerate wound healing and tissue regeneration;
- b. enhance the performance of skin grafts;
- c. improve the attachment of gum tissue to the tooth surface;
- d. improve the biocompatibility of medical devices; and
- e. accelerate cell proliferation,

15 by providing an amount effective of r-laminin 5 for the various methods. The invention also provides methods and kits for using laminin 5 to regulate angiogenesis. The kits comprise an amount of laminin 5 or r-laminin 5 effective for the desired effect, and instructions for the use 20 thereof.

In a further aspect, the present invention provides improved medical devices and grafts, wherein the improvement comprises coating the devices or grafts with an amount effective of r-laminin 5 or the pharmaceutical compositions of the invention for the desired application.

25 In a further aspect, the invention provides improved cell culture devices for the proliferation of cells in culture, by providing an amount effective of r-laminin 5 for the attachment of cells to a cell culture device for the attachment and subsequent proliferation, differentiation, or maintenance of the cells.

30 In another aspect, the invention provides a cell culture growth supplement, comprising r-laminin 5. In another aspect, the invention provides an improved cell culture growth media, wherein the improvement comprises the addition of r-laminin 5.

Brief Description of the Figures

Figure 1 is a bar graph showing the results of an HFK cell adhesion assay for r-laminin 5 activity in culture media from various clones.

Figure 2 is a bar graph showing a cell adhesion assay in which r-laminin 5 was coated directly onto the plate. p1b5 = anti-integrin $\alpha 3\beta 1$ antibody; sp2 = control IgG, non-specific; C29: anti-laminin 5 antibody

Figure 3 is a rotary shadow analysis of r-laminin 5. Purified r-laminin 5 protein was diluted to 50 μ g/ml and adjusted to 70% glycerol/30% 0.15M ammonium bicarbonate and rotary shadowed using standard techniques. An approximately 80,000X magnification field is shown of (A) r-laminin 5; (B) "native" laminin 5 (purified by BM165 monoclonal antibody affinity chromatography from SCC-25 (squamous cell carcinoma cell line) conditioned medium). The bar represents 50 nm.

Detailed Description of the Preferred Embodiments

All references, patents and patent applications are hereby incorporated by reference in their entirety.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein "laminin 5" encompasses both r-laminin 5 and heterotrimeric laminin 5 from naturally occurring sources.

The term "r-laminin 5" refers to include recombinant heterotrimeric laminin 5 expressed by a cell that has been exogenously transfected with expression vector(s) comprising polynucleotides that encode $\alpha 3$, $\beta 3$ and $\gamma 2$ laminin polypeptide chains, or a portion of each of the

chains which are capable of forming a heterotrimeric laminin 5, as well as versions thereof resulting from cellular processing events. Such r-laminin 5 can comprise α 3, β 3, and γ 2 sequences from a single organism, or from different organisms. Laminin 5 chain DNA sequences and their encoded proteins from a variety of organisms are known in the art. (See, for
5 example, Gerecke et al., J. Biol. Chem. 269:11073-11080 (1994); Kallunki et al., J. Cell Biol. 119:679-693 (1992); Ryan et al., J. Biol. Chem. 269:22779-22787 (1994); Iivananinen et al., J. Biol. Chem. 274:14107-14111 (1999); Galliano et al., J. Biol. Chem. 270:21820-221826 (1995); Sugiyama et al., Eur. J. Biochem. 228:120-128 (1995) all references incorporated by reference herein in their entirety).

10 In the present invention, r-laminin 5 is a secreted protein, which is capable of being directed to the ER, secretory vesicles, and the extracellular space as a result of a signal sequence, as well as those proteins released into the extracellular space without necessarily containing a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Such processing event can be variable, and thus may yield different versions of the final "mature protein". The
15 substantially purified r-laminin 5 of the present invention includes heterotrimers comprising both the full length and any such processed laminin 5 chains.

As used herein, the term "substantially purified" means that the recombinant laminin 5 so designated has been separated from its in vivo cellular environments.

20 As used herein, a laminin 5 polypeptide chain refers to a polypeptide chain according to one or more of the following:

(a) comprises a polypeptide structure selected from the group consisting of:

1. R1-R2-R3
2. R1-R2-R3(e)
3. R3
4. R3(e)
5. R1-R3
6. R1-R3(e)
7. R2-R3
8. R2-R3(e)

wherein R1 is a amino terminal methionine; R2 is a signal sequence that is capable of directing secretion of the polypeptide, wherein the signal sequence may be the natural signal sequence for the particular laminin chain, that of another secreted protein, an artificial sequence; R3 is a secreted laminin chain selected from the α 3, β 3, and γ 2 chains; and R3(e) is a 5 secreted laminin chain selected from the α 3, β 3, and γ 2 chains that further comprises an epitope tag (such as those described below), which can be placed at any position within the laminin chain amino acid sequence; and/or

10 (b) is encoded by a polynucleotide that is substantially similar to the disclosed laminin polynucleotide sequences or portions thereof (SEQ ID NOS.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35); and/or

15 (c) is encoded by a polynucleotide that hybridizes under high or low stringency conditions to coding regions, or portions thereof, of one or more of the recombinant laminin 5 chain DNA sequences disclosed herein (SEQ ID NOS.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35), or complementary sequences thereof; and/or

20 (d) has at least 70% identity to the disclosed laminin polypeptide claim amino acid sequences (SEQ ID NOS.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36), preferably at least 80% identity, and most preferably at least about 90% identity.

The phrase "substantially similar" is used herein in reference to polynucleotide or polypeptide sequences having one or more conservative variations from the laminin 5 sequences disclosed herein, including but not limited to deletions, insertions, inversions, repeats, and substitutions, wherein the resulting laminin chain is functionally equivalent to those disclosed herein.

25 For example, conservative polynucleotide variants may contain alterations in the coding regions, non-coding regions, or both. Especially preferred are polynucleotide variants containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred. Moreover, variants in which 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination are also preferred. Polynucleotide variants can be produced for a variety of reasons, including but 30 not limited to optimizing codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

Naturally occurring conservative variants are called "allelic variants," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985).) These allelic variants can vary at either the polynucleotide and/or polypeptide level. Alternatively, non-naturally occurring 5 conservative variants may be produced by mutagenesis techniques or by direct synthesis.

Using known methods of protein engineering and recombinant DNA technology, conservative polynucleotide variants may be generated to improve or alter the characteristics of the expressed laminin chain polypeptides. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the secreted protein. (See, for example, Ron et al., J. Biol. 10 Chem. 268: 2984-2988 (1993); Dobeli et al., J. Biotechnology 7:199-216 (1988)) Ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. (See, for example, Gayle et al., J. Biol. Chem 268:22105-22111 (1993)) Furthermore, even if deleting one or more amino acids from the N-terminus or C-terminus of a polypeptide results in modification or loss of one or more biological functions, 15 other biological activities may still be retained.

Guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. et al., Science 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

The first strategy exploits the tolerance of amino acid substitutions by natural selection 20 during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological 25 activity of the protein.

The second strategy uses genetic engineering to introduce amino acid changes at specific 30 positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used. (Cunningham and Wells, Science 244:1081-1085 (1989).) The resulting mutant molecules can then be tested for biological activity.

As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried

(within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Moreover, tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of 5 the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

The "substantially similar" polypeptides of the present invention also include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted 10 amino acid residues may or may not be one encoded by the genetic code, or (ii) substitution with one or more of amino acid residues having a substituent group; (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol); and/or (iv) fusion of the 15 polypeptide with additional amino acids, such as an IgG Fc fusion region peptide, or leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both 20 reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).)

"Stringency of hybridization" is used herein to refer to conditions under which nucleic acid hybrids are stable. The invention also includes nucleic acids that hybridize under high 25 stringency conditions (as defined herein) to all or a portion of the coding sequences of the laminin chain polynucleotides disclosed herein, or their complements. The hybridizing portion of the hybridizing nucleic acids is typically at least 50 nucleotides in length. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_M) of the hybrids. T_M decreases approximately 1-1.5°C with every 1% decrease in sequence homology. In 30 general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to

such washing conditions. Thus, as used herein, high stringency refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at 5 about 65°C.

Also contemplated are laminin 5-encoding nucleic acid sequences that hybridize to the polynucleotides of the present invention at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered 10 stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH₂PO₄; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 ug/ml salmon sperm blocking DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher 15 salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The 20 inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

As used herein, "percent identity" of two amino acids or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 25). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to obtain an amino acid sequence homologous to 30 a polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids. Res. 25:3389-3402, 1997).

When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See <http://www.ncbi.nlm.nih.gov>.

Further embodiments of the present invention include polynucleotides encoding laminin chain polypeptides having at least 70% identity, preferably at least 80% identity, and most 5 preferably at least 90% identity to one or more polypeptide sequences, or fragments thereof, contained in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

As used herein, " α 3 polynucleotide" refers to polynucleotides encoding an α 3 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) the nucleotides of said polynucleotide may encode an amino acid sequence 10 substantially similar to one or more of the sequences set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12 or fragments thereof, or fragments thereof; (b) polynucleotides that encode polypeptides which share at least 70% identity, preferably 80% identity, and most preferably at least 90% identity with one or more of the sequences set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12 or fragments thereof; (c) the α 3 polynucleotides hybridize under low or high stringency conditions to the 15 coding sequence set forth in one or more of SEQ ID NO: 1, 3, 5, 7, 9, 11, fragments thereof, or complementary sequences thereof; (d) the α 3 polynucleotides may encode a polypeptide with a general structure selected from (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted α 3 chain polypeptides.

As used herein, " β 3 polynucleotide" refers to polynucleotides encoding a β 3 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) the nucleotides of said polynucleotide may encode an amino acid sequence 20 substantially similar to one or more of the sequences set forth in SEQ ID NO: 14, 16, 18, 20, 22, 24, or fragments thereof; (b) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with one or more of the sequences set forth in SEQ ID NO: 14, 16, 18, 20, 22, 24, or fragments thereof; (c) the β 3 polynucleotides hybridize under low or high stringency conditions to the coding sequence of one 25 or more of the sequences set forth SEQ ID NO: 13, 15, 17, 19, 21, 23, fragments thereof or complementary sequences thereof; (d) the β 3 polynucleotides may encode a polypeptide with a general structure selected from (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3;

(6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted β 3 chain polypeptides.

As used herein, " γ 2 polynucleotide" refers to polynucleotides encoding a γ 2 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following:

- 5 (a) the nucleotides of said polynucleotide may encode an amino acid that is substantially similar to one or more of the sequences set forth in SEQ ID NO: 26, 28, 30, 32, 34, 36 or fragments thereof; (b) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with one or more of the sequences set forth in SEQ ID NO: 26, 28, 30, 32, 34, 36 or fragments thereof; (c) the γ 2 polynucleotides hybridize under low or high stringency conditions to the coding sequence set forth in one or more of SEQ ID NO: 25, 27, 29, 31, 33, 35, fragments thereof, or complementary sequences thereof; (d) the γ 2 polynucleotides may encode a polypeptide with a general structure selected from (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted γ 2 chain polypeptides.
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- 15

As used herein, the term "epitope tag" refers to a polypeptide sequence that is expressed as part of a chimeric protein, where the epitope tag serves as a recognition site for binding of antibodies generated against the epitope tag, or for binding of other molecules that can be used for affinity purification of sequences containing the tag.

- 20 As used herein, the term "increased biocompatibility" refers to reduced induction of acute or chronic inflammatory response, and reduced disruption of the proper differentiation of implant-surrounding tissues for laminin 5-coated biomaterials relative to an analogous, non-coated biomaterial.

As used herein the term "graft" refers to both natural and prosthetic grafts and implants.

- 25 In one aspect, the present invention provides cells that have been systematically transfected with recombinant expression vectors comprising promoter sequences that are operatively linked to polynucleotide sequences encoding polypeptide sequences comprising α 3, β 3, and γ 2 laminin 5 chains. After the multiple transfections, the cells express each of the recombinant laminin 5 chains, which assemble into a heterotrimer and can be purified from the
- 30 cell culture medium.

In a preferred embodiment, cDNAs encoding proteins comprising the α 3, β 3, and γ 2 laminin polypeptide chains, or fragments thereof, are subcloned into an expression vector. Alternatively, laminin 5 α 3, β 3, and/or γ 2 gene sequences, including one or more introns, and including various 5' and 3' non-coding regions, can be used.

5 Any cell capable of expressing and secreting the r-laminin 5 can be used. Preferably, eukaryotic cells are used, and most preferably mammalian cells are used, including but not limited to kidney and epithelial cell lines. Especially preferred are those mammalian cells that do not endogenously express laminin 5. Carbohydrate and disulfide post-translational modifications are believed to be required for laminin 5 protein folding and function. This makes
10 10 the use of eukaryotic cells preferable for producing functional r-laminin 5, although other systems are useful for obtaining, for example, antigens for antibody production.

"Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the laminin 5 individual chains may be
15 15 constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the
20 20 invention is intended to include other expression vectors that serve equivalent functions, such as viral vectors.

In one embodiment, at least one of the laminin chain polypeptide sequences, or fragments thereof, is operatively linked to a nucleic acid sequence encoding an "epitope tag", so that at least one of the chains is expressed as a fusion protein with an expressed epitope tag. The
25 25 epitope tag may be expressed as the amino terminus, the carboxy terminus, or internal to the end of a r-laminin 5 chain, so long as the resulting heterotrimeric r-laminin 5 remains functional. Any epitope tag may be utilized, so long as it can be used as the basis for affinity purification of the resulting r-laminin 5 heterotrimer. Examples of such epitope tags include, but are not limited to FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His
30 30 (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Manheim Biochemicals).

In another embodiment, one of the r-laminin 5 chains is expressed as a fusion protein with a first epitope tag, and at least one other r-laminin chain is expressed as a fusion protein with a second epitope tag. This permits multiple rounds of purification to be carried out. Alternatively, the same epitope tag can be used to create fusion proteins with more than one of

5 the r-laminin chains.

In a further embodiment, the epitope tag can be engineered to be cleavable from the r-laminin 5 chain(s). Alternatively, no epitope tag is fused to any of the r-laminin 5 chains, and the r-laminin 5 is purified by standard chromatography techniques, including but not limited to affinity chromatography using laminin 5 specific antibodies or other laminin 5 binding

10 molecules, ionic exchange, hydrophobic exchange, etc.

Transfection of expression vectors into the host cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformation, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection.

15 In a preferred embodiment, the cells are stably transfected. Any methods for stable transfection and selection of appropriate transfected cells are known in the art. In a most preferred embodiment, a CMV promoter driven expression vector is used in a human kidney embryonic 293 cell line.

Media from cells transfected with a single laminin chain are initially analyzed on
20 Western blots using chain-specific anti-laminin-5 antibodies. The expression of single laminin chains following transfection is generally intracellular. Clones showing reactivity against individual transfected chain(s) are verified by any appropriate method, such as PCR, reverse transcription-PCR, or nucleic acid hybridization, to confirm incorporation of the transfected gene. Preferably, analysis of genomic DNA preparations from such clones is done by PCR using
25 laminin chain-specific primer pairs. Media from transfected clones producing all three chains are further analyzed for heterotrimeric laminin 5 secretion and/or activity, by any appropriate method, including Western blot analysis and cell binding assays, such as a keratinocyte cell adhesion assay.

In another aspect, the present invention provides substantially purified r-laminin 5,
30 comprising an α_3 chain, a β_3 chain, and a γ_2 chain, and methods for producing substantially purified r-laminin 5. In one embodiment, the r-laminin 5 comprises a first chain comprising a

polypeptide that is substantially similar to at least one of the sequences shown in SEQ ID NO:2, 4, 6, 8, 10, 12 or fragments thereof; a second chain comprising a polypeptide that is substantially similar to at least one of the sequences shown in SEQ ID NO:14, 16, 18, 20, 22, 24, or fragments thereof; and a third chain comprising a polypeptide that is substantially similar to at least one of the sequences shown in SEQ ID NO:26, 28, 30, 32, 34, 36, or fragments thereof, wherein the first, second, and third polypeptides are produced recombinantly, and wherein the first, second, and third chains assemble into a recombinant heterotrimeric laminin 5.

In another embodiment, the substantially purified r-laminin 5 comprises a first chain comprising a polypeptide that is at least about 70% identical to at least one of the sequences shown in SEQ ID NO:2, 4, 6, 8, 10, 12, or fragments thereof; a second chain comprising a polypeptide that is at least 70% identical to at least one of the sequences shown in SEQ ID NO:14, 16, 18, 20, 22, 24, or fragments thereof; and a third chain comprising a polypeptide that is at least 70% identical to at least one of the sequences shown in SEQ ID NO:26, 28, 30, 32, 34, 36, or fragments thereof, wherein the first, second, and third polypeptides assemble into a recombinant heterotrimeric laminin 5.

In a preferred embodiment, at least one of the first, second, or third chains of the substantially purified human r-laminin 5 is expressed as a fusion protein with an epitope tag.

Alternatively, the r-laminin 5 comprises a heterotrimeric polypeptide structure, wherein each individual chain comprises a general structure selected from the group consisting of: (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e)

wherein R1 is a amino terminal methionine; R2 is a signal sequence that is capable of directing secretion of the polypeptide, wherein the signal sequence may be the natural signal sequence for the particular laminin chain, that of another secreted protein, or an artificial sequence; R3 is a secreted α 3, β 3, or γ 2 laminin chain; and R3(e) is a secreted laminin α 3, β 3, and γ 2 chain that further comprises an epitope tag (such as those described above), which can be placed at any position within the laminin chain amino acid sequence.

In a preferred embodiment, purification of the r-laminin 5 is accomplished by passing media from the transfected cells through an affinity column. For example, antibodies or other binding molecules that bind to a peptide epitope expressed on at least one of the recombinant chains are attached to an affinity column, and bind r-laminin 5 that has been secreted into the

media. The r-laminin 5 is removed from the column by passing excess peptide through the column. The eluted protein can subsequently be further purified, if desired.

Eluted fractions are analyzed by any appropriate method, including gel electrophoresis and Western blot analysis. In a further embodiment, the peptide epitope can be cleaved after 5 purification. In other embodiments, two or three separate r-laminin chains are expressed as fusion proteins, each with a different epitope tag, permitting two or three rounds of purification and a doubly or triply purified r-laminin 5. The epitope tag can be engineered so as to be cleavable from the r-laminin 5 chain(s) after purification. Alternatively, no epitope tag is fused 10 to any of the r-laminin 5 chains, and the r-laminin 5 is purified by standard techniques, including but not limited to affinity chromatography using laminin 5 specific antibodies or other laminin 5 binding molecules.

In another aspect, the present invention provides novel laminin β 3 and γ 2 chain nucleic acids and proteins, consisting of the nucleic acid sequences and proteins disclosed as SEQ ID NO:21-22, 23-24, 29-30, and 31-32.

15 The present invention further provides pharmaceutical compositions comprising r-laminin 5, as disclosed above, and a pharmaceutically acceptable carrier. According to this aspect of the invention, other agents can be included in the pharmaceutical compositions, depending on the condition being treated, including but not limited to any of the collagens, other laminin types, fibronectin, integrins, glycoproteins, proteoglycans, heparan and heparan sulfate 20 proteoglycans; growth factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and keratinocyte growth factor (KGF); glycosaminoglycans, entactin, nidogen, and peptide fragments thereof.

Pharmaceutical preparations comprising r-laminin 5 can be prepared in any suitable form, and generally comprise the r-laminin 5 in combination with any of the well known 25 pharmaceutically acceptable carriers. The carriers can be injectable carriers, topical carriers, transdermal carriers, and the like. The preparation may advantageously be in a form for topical administration, such as an ointment, gel, cream, spray, dispersion, suspension or paste. The preparations may further advantageously include preservatives, antibacterials, antifungals, antioxidants, osmotic agents, and similar materials in composition and quantity as is 30 conventional. Suitable solutions for use in accordance with the invention are sterile, are not harmful for the proposed application, and may be subjected to conventional pharmaceutical

operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton, Pa. (1975).

5 The dosage regimen for various treatments using the r-laminin 5 of the present invention is based on a variety of factors, including the type of injury or condition, the age, weight, sex, medical condition of the individual, the severity of the condition, and the route of administration. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Laminins are extremely potent molecules, and one or a few molecules per
10 cell could produce an effect. Thus, effective doses in the pico-gram per milliliter range are possible if the delivery is optimized. Laminins are sometimes present in an insoluble form in the basement membrane and have the capability of polymerizing at concentrations ranging as low as about 50 µg/ml, depending on the laminin isoform and the conditions. Laminins can also polymerize into a gel at a concentration of 2-3 mg/ml. Dosage levels of the order of between 1
15 ng/ml and 10 mg/ml are thus useful for all methods disclosed herein, preferably between about 1 µg/ml and about 3 mg/ml.

The treatment regime will also vary depending on the condition of the subject, based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, and the route of administration. For example, r-laminin
20 5 can be used to coat a wound dressing, which is placed in contact with a patient's wound as frequently as the dressing needs to be changed, and for as long as the dressing is applied to the wound surface.

Similarly, the route of administration will vary depending on the condition of the subject, based on a variety of factors, including the type of injury, the age, weight, sex, medical condition
25 of the individual, and the severity of the condition.

In further aspect, the present invention provides methods for using r-laminin 5, or the pharmaceutical compositions of the invention, to accelerate wound healing and tissue regeneration. In preferred embodiments, r-laminin 5 is used to accelerate the healing of skin in diabetic foot ulcers, venous ulcers, pressure sores, skin surgery, severe burns, and acute wounds,
30 and enhanced performance of skin grafts (both autologous and artificial). In another aspect, the

present invention provides kits for carrying out these methods, comprising an amount effective of laminin 5 or r-laminin 5 and instructions for using the laminin 5 to carry out the methods.

In one embodiment, r-laminin 5, or a pharmaceutical composition comprising r-laminin 5, is used to enhance wound healing by promoting the adhesion of transplanted cultured keratinocytes or other epithelial cells to an underlying substrate, such as a mammalian or human dermis. The substrate may comprise a wound surface, the basal surface of a confluent layer of cultured epithelial cells to be transplanted, or a substrate to be applied to the wound surface, such as a wound dressing, prior to placing the layer on a graft site. The r-laminin 5 may be supplied in a pharmaceutically acceptable carrier, preferably in amounts of between about 1 ng/ml and 10 about 10 mg/ml.

The use of kalinin-containing (ie: laminin 5-containing) isolated cell matrices has previously been shown to enhance the adhesion of transplanted cultured keratinocytes to an underlying substrate (Burgeson et al., US Patent No. 5,770,562). This and other studies have thus demonstrated that laminin 5 stimulates epithelial cell attachment and spreading, and thus provides an appropriate surface facilitating the healing of skin and the use of skin grafts. (Quaranta and Hormia, U.S. Patent No. 5,422,264; Jones, U.S. Patent No. 5,541,106; Quaranta and Hormia, U.S. Patent No. 5,658,789; Hormia et al., J. Invest. Dermatol. 1995 Oct. 105(4):557-561; Takeda et al., J. Invest. Dermatol. 1999 Jul; 113(1):38-42; Goldfinger et al., J. Cell Sci. 1999; 112(Pt. 16):2615-2629).

Thus, the addition of r-laminin 5 to the appropriate injured tissue can promote cell growth, cell migration, and accelerate tissue regeneration. Accelerated healing has the added benefit of reducing inflammatory responses and scarring. This can be accomplished in some cases by simply coating the r-laminin 5 or the pharmaceutical compositions of the invention into a wound area (such as skin, periodontal epithelial cells), or in other cases, by providing a suitable substrate to which r-laminin 5 has been anchored, including but not limited to wound dressing and matrices, graft substrates, and dental abutments.

The incorporation of recombinant r-laminin 5 into wound repair dressings and matrices as well as tissue grafts will provide a natural ligand interactive surface to promote normal cell adherence, cell growth and tissue development. Many grafts are used to replace tissue that has an epithelial cell layer adherent to a basal lamina. When an inappropriate surface is provided to these cells following grafting, the graft is at risk for failure of restoration of the normal cell

layer. The advantage of coating these grafts with r-laminin 5 is to create a surface that sufficiently recapitulates a normal basal lamina surface to promote cell re-population.

Skin grafts are used in cases where large surface areas of skin have been burned or injured. The application of r-laminin 5 and/or the pharmaceutical compositions of the invention will significantly promote the attachment and 'take' of skin grafts to the injured tissue, as well as promote normal skin healing processes while minimizing scar tissue formation.

Collagen-based matrices are also applied to serious skin injuries to promote the growth of the underlying dermis and improve the take of a skin graft. Coating the collagen matrices with r-laminin-5 will create a more natural ligand interactive surface to promote cell migration, cell proliferation and the regeneration of the dermis. An acceleration of the regeneration of the dermis, and take of the skin graft, will minimize scar tissue formation.

Purified laminin 5 has been demonstrated to support epithelial cell adhesion to the internal basal lamina of teeth (Mullen et al., J. Periodontal. Res. 1999 Jan 34(1):16-24; Hormia et al., J. Dent. Res. 1998 Jul; 77(7):1479-1485) and is believed to strengthen the anchorage of ameloblasts (ie: enamel-producing cells) to the enamel matrix. (Yoshiba et al., Cell Tissue Res. 1998 Apr; 292(1):143-149). Thus, in another embodiment, the r-laminin 5 or the pharmaceutical compositions of the invention are used to stimulate epithelium cell adhesion to the internal basal lamina of teeth and of ameloblasts to the enamel matrix of teeth. Such treatments are useful for the treatment of periodontal diseases, including but not limited to oral ulcerations, gingivitis and periodontitis. For example, existing teeth may be coated with the r-laminin 5 or the pharmaceutical compositions of the present invention as a treatment for gum (junctional epithelium) diseases, including but not limited to gingivitis and periodontitis, which promote the detachment of the gum from the tooth. These disease conditions allow the accumulation of food and other foreign matter in the space between the gum and the tooth, resulting in infection. The r-laminin 5 will promote reattachment of the gum to the tooth, thus preventing entry of foreign matter and subsequent infection.

For use in treating gingivitis and other periodontal diseases and disorders, the pharmaceutical compositions of the present invention may be in the form of toothcreams, toothpastes, liquid dentifrices, tooth-powders chewing-gum, tablets and the like. The pharmaceutical compositions of the invention can also contain flavoring, coloring agents, sweeteners, preservatives, surface active agents, and the like.

Purified laminin-5 has been shown to promote the *in vitro* expansion of epithelial cells (Gonzales et al., Mol. Biol. Cell. 1999 Feb; 10(2):259-270; Baker et al., Exp. Cell Res. 1996 Nov 1; 228(2):262-270), pancreatic beta islet cells (Todorov et al., Transplant. Proc. 1998 Mar; 30(2): 455; Quaranta and Jones, U.S. Patent No. 5,510,263; Halberstadt et al., U.S. Patent No. 5,681,587; Halberstadt et al., U.S. Patent No. 5,672,361), and T cells (Vivinus-Nebot et al., J. Cell Biol. 1999 Feb 8; 144(3):563-574), by providing an efficient adhesion substrate for primary cell cultures. Thus, in another aspect of the present invention, r-laminin 5 is used to enhance the adhesion of cells for proliferation, differentiation, or maintenance of cells including, but not limited to pancreatic beta islet cells, epithelial cells, or T cells, by contacting the cells with an amount effective of r-laminin 5 to provide an efficient adhesion substrate for attachment and subsequent proliferation, differentiation, or maintenance of the cells. The r-laminin 5 can be provided in the cell culture medium, as a cell culture medium supplement, or may be coated on the surface of a cell growth substrate. In each case, r-laminin 5 is preferably used at a concentration of between about 1 ng/ml and about 10 mg/ml. The cells can optionally be contacted with other compounds that promote cell adhesion, proliferation, differentiation, and/or maintenance, including but not limited to any of the collagens, other laminin types, fibronectin, integrins, glycoproteins, proteoglycans, heparan sulfate proteoglycan, glycosaminoglycans, entactin, nidogen, and peptide fragments thereof.

The cells may be primary cells or cell lines. The methods of this aspect of the invention can be used *in vivo*, *ex vivo*, or *in vitro*.

In a preferred embodiment, r-laminin 5 is used to coat the surface of a substrate to promote cell adhesion to the substrate, and to stimulate cell proliferation, differentiation, and/or maintenance. The substrate used herein may be any desired substrate. For laboratory use, the substrate may be as simple as glass or plastic. For use *in vivo*, the substrate may be any biologically compatible material capable of supporting cell growth. Suitable substrate materials include shaped articles made of or coated with such materials as collagen, regenerated collagen, polyglycolic acid, polygalactose, polylactic acid or derivatives thereof; biocompatible metals such as titanium and stainless steel; ceramic materials including prosthetic material such as hydroxylapatite; synthetic polymers including polyesters and nylons; polystyrene; polyacrylates; polytetrafluoroethylene, and virtually any other material to which biological molecules can

readily adhere. The determination of the ability of a particular material to support adhesion of r-laminin 5 of the invention requires only routine experimentation by the skilled artisan.

In a further aspect, the present invention provides a method of treating Type I diabetes in a patient in need thereof comprising contacting pancreatic beta islet cells with an amount effective of r-laminin 5 to provide an efficient adhesion substrate for the cells, leading to increased proliferation of insulin-producing pancreatic beta islet cells, and administering the cells to a patient in need thereof.

Nearly two million Americans are afflicted with Type I (insulin-dependent) diabetes, in which the pancreas has lost its ability to secrete insulin due to an autoimmune disorder in which 10 the insulin-secreting beta cells, found within the islet cells of the pancreas, are destroyed. Although insulin injections can compensate for beta cell destruction, blood sugar levels can still fluctuate dramatically. The impaired ability to take up glucose from the blood results in side reactions in which toxic products accumulate, leading to complications including blindness, kidney disease, nerve damage, and, ultimately, coma and death. (U.S. Patent No. 5,672,361)

15 The pancreatic beta islet cells to be grown are plated on or applied to the matrix-coated substrate using standard tissue culture techniques, followed by expansion in standard cell growth medium (as disclosed in U.S. Patent No. 5,672,361) in the presence of r-laminin 5. Any medium capable of supporting the enhanced growth of adult islet cells on the matrix-coated substrate is within the scope of the invention, as discussed above.

20 Fetal pancreatic islet cells may be grown in vitro in the presence of r-laminin 5 for transplantation into diabetic patients. Growth of fetal pancreatic islet cells in the presence of r-laminin 5 increases the yield of islet cells for transplantation and thus solves the need to produce larger amounts of these cells. In addition, it is contemplated that the inclusion of other growth factors in the adult islet cell culture medium will further increase the yield of islet cells.

25 Laminins, or cell extracts containing laminins have been shown to regulate angiogenesis in a biphasic manner. (See, for example, Nicosia et al., Dev. Biol. 164:197-206 (1994); Bonfil et al., Int. J. Cancer 58:233-239 (1994)). At lower concentrations (30-300 µg/ml), a laminin-entactin complex stimulated angiogenesis in a three-dimensional culture, while at 3000 µg/ml the same complex was inhibitory to angiogenesis. Thus, in another aspect, the present invention 30 provides methods for regulating angiogenesis, comprising contacting a tissue or culture substrate with an amount effective of laminin 5 or pharmaceutical compositions thereof to regulate

angiogenesis. In one embodiments, the laminin 5 is used to promote angiogenesis by contacting a tissue or culture substrate with an amount effective of laminin 5 to promote angiogenesis. In another embodiment, the laminin 5 is used to inhibit angiogenesis, by contacting the tissue or culture substrate with an amount effective of laminin 5 to inhibit angiogenesis. An example of 5 culture substrates to be contacted with laminin 5 to regulate angiogenesis are those used for tissue engineering purposes.

As used herein, the term "angiogenesis" refers to the formation of blood vessels. Specifically, angiogenesis is a multistep process in which endothelial cells focally degrade and invade through their own basement membrane, migrate through interstitial stroma toward an 10 angiogenic stimulus, proliferate proximal to the migrating tip, organize into blood vessels, and reattach to newly synthesized basement membrane (see Folkman et al., *Adv. Cancer Res.*, Vol. 43, pp. 175-203 (1985)). Compounds that promote angiogenesis can be used to promote wound healing and skin grafting, organ transplantation (including artificial organs), acceleration of endothelial cell coverage of vascular grafts to prevent graft failure due to re-occlusion, to treat 15 ischemic conditions, and to treat inflammatory diseases.

In a further aspect, the present invention provides cell substrates comprising an amount effective of r-laminin 5 for the adhesion, growth, or maintenance of cells in culture,. The substrates may comprise any of the substrates discussed above. Preferably, the r-laminin 5 is coated on the surface of the substrate using solution at a concentration of between about 1 ng/ml 20 and about 10 mg/ml.

In another aspect of the present invention, an improved cell culture medium is provided, wherein the improvement comprises addition to the cell culture medium of an effective amount of r-laminin 5 to the cell culture medium to promote the adherence, proliferation, and/or maintenance of cells. Any cell culture media that can support the growth of cells can be used 25 with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, RPMI Medium, and Macrophage-SFM Medium or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or 30 non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture

media is commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO). Alternatively, the r-laminin 5 is used as a cell culture supplement, and can be separately added to the cell culture medium.

Purified laminin-5 has also been shown to promote epithelial cell attachment to a wide
5 variety of biomaterials, including polymers, hydroxyapatite, and metals, thus improving the biocompatibility of the biomaterials. (Jones et al., U.S. Patent No. 5,585,267; El Ghannam et al., J. Biomed. Mater. Res. 1998 Jul; 41(1):30-40)

Thus, in a further aspect, the present invention comprises medical devices with improved
10 biocompatibility, wherein the devices are coated with the r-laminin 5 of the invention, alone or in combination with other proteins or agents that serve to increase the biocompatibility of the device surface. The coated device stimulates cell attachment and provides for diminished inflammation and/or infection at the site of entry of the appliance. The device may also be used to stimulate gum junctional epithelium adhesion in the treatment of gingivitis and periodontitis.

Preferably, the device is a shaped article that is either an indwelling or transcutaneous
15 catheter, polytetrafluoroethylene (PTFE), expanded PTFE (EPTFE), needle, metal pin, metal rod, colostomy tube, transcutaneous catheter, dental abutment piece or surgical mesh. In another aspect of this preferred embodiment, the device is used *in vivo*. Preferably, the appliance is made of or coated with a biocompatible metal that may be either stainless steel or titanium. Alternatively, the device is made of or coated with a ceramic material, or a polymer including
20 but not limited to polyester, polyglycolic acid or a polygalactose-polyglycolic acid copolymer.

One particular use of the present invention is to increase epithelial cell adhesion to target surfaces. For example, prostheses for dental implantation may be coated with the r-laminin 5 of the invention to stimulate periodontal cell attachment. These prostheses typically comprise two separate pieces, an implant which is inserted into the bone and an abutment piece which actually
25 contacts the junctional epithelium. Alternatively, the implant and abutment piece may be obtained as a single unit.

If the device is made of a natural or synthetic biodegradable material in the form of a mesh, sheet or fabric, the r-laminin 5 may be applied directly to the surface thereof. Epithelial cells may then be cultured on the matrix to form transplantable or implantable devices, including
30 dental abutment pieces, needles, metal pins or rods, indwelling catheters, colostomy tubes, surgical meshes and any other appliance for which coating with the r-laminin is desirable.

Alternatively, the devices may be implanted and cells may be permitted to attach in vivo. The epithelial cell-coated surgical meshes will be useful for skin allografts necessitated by compromised skin integrity.

Coupling of the r-laminin 5 may be non-covalent (such as by adsorption), or by covalent means. The device may be immersed in, incubated in, or sprayed with the r-laminin 5 of the invention. In a preferred embodiment, the concentration of r-laminin 5 for coating the device is between about 1 ng/ml and about 10 mg/ml.

The present invention also provides a method for inducing epithelial cell attachment to the device (as disclosed above), comprising coating the appliance with r-laminin 5 prior to 10 incubation with epithelial cells.

The therapeutic application of r-laminin 5 produced in accordance with the present invention can be used for the treatment of a variety of conditions and diseases, including but not limited to Type I diabetes; skin conditions including but not limited to diabetic foot ulcers, venous ulcers, pressure sores, skin surgery, burns, acute wounds, and skin grafts; corneal 15 ulcerations; gastro-intestinal ulcers; periodontitis; and gingivitis. The therapeutically effective amount of r-laminin 5 for use in these conditions and diseases can be readily ascertained by one of ordinary skill in the art.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit 20 the scope of the invention, as defined by the claims appended hereto.

Examples

Production of r-laminin-5 involved sequential transfections of a mammalian cell line with vectors containing cDNAs that encode for the chains of the laminin-5 molecule, namely α_3 , β_3 25 and γ_2 . An additional polynucleotide sequence that encodes the 'flag' peptide (DYKDDDDK), was added to the amino terminus end of the β_3 gene to facilitate affinity purification of the expressed heterotrimeric recombinant laminin-5 molecule.

IV. Materials and Methods

Expression vector constructs for $\alpha 3$

The entire coding sequence of the $\alpha 3$ cDNA [SEQ ID NO:1] was cloned via standard techniques into the expression vector pcDNA3.1/Zeo (Invitrogen), which contains the Zeocin resistant gene for selection. The expression vectors were used to produce stable cell lines according to the manufacturer's instructions.

In order to produce a second $\alpha 3$ expression vector, the full-length $\alpha 3$ cDNA was excised from the pZeoo $\alpha 3$ expression construct by digestion with KpnI-NotI restriction enzymes. The double digested $\alpha 3$ fragment was inserted in the expression vector pTargeT (Promega; Madison, WI), generating pTgTa $\alpha 3$. This expression construct carries the G418 resistant gene for selection of resistant clones. Both expression constructs have been analyzed by restriction enzyme mapping and DNA sequencing.

Construction of full-length $\beta 3$ chain

Two cDNA fragments, Kal5-5c and Kal92-1, each cloned into separate pCR II vectors (Invitrogen), which together encode the entire $\beta 3$ chain of laminin-5 [SEQ ID NO:19], were received from Dr. Burgeson's laboratory (4). The two fragments were cloned into a single vector to obtain the full-length $\beta 3$ chain, plasmid PCRII $\beta 3$.

Expression vector constructs for $\beta 3$

The laminin $\beta 3$ expression vector, pRCX3 $\beta 3_F$, was constructed containing the full-length $\beta 3$ chain obtained for pCRII $\beta 3$ and the FLAG epitope added to the amino terminus [SEQ ID NO:17-18]. pRCX3 is a vector derived from pRC/CMV (Invitrogen) and it contains a Geneticin resistant gene for selection with G418 sulfate, a BM 40 (SPARC) signal peptide sequence and the Flag peptide sequence in frame with convenient cloning sites

A second $\beta 3$ expression vector was constructed by excising the complete laminin $\beta 3$ -flag peptide coding region from pRCX3 $\beta 3_F$ plasmid and introducing it into pcDNA3.1/Zeo. This expression constructs carries the Zeocin resistant gene for selection.

Both $\beta 3$ -expression constructs have been analyzed by restriction enzyme mapping and DNA sequencing.

Expression vector constructs for $\gamma 2$

The full-length $\gamma 2$ cDNA [SEQ ID NO:29] was excised from pVL1393 $\gamma 2$ (received from Dr. Karl Tryggvason, Karolinska Institute, Sweden) by digestion with BamH I-Xba I restriction enzymes. The double digested $\gamma 2$ fragment was inserted in the corresponding sites of the expression vector pcDNA3.1/Zeo (Invitrogen), generating the pZeoy2 expression construct. This expression constructs carries the Zeocin resistant gene for selection.

Similarly, a BamH I-Not I full-length $\gamma 2$ cDNA fragment was cloned into the expression vector pTargeT (Promega), generating pTgTy2. This expression construct carries the G418 resistant gene for selection of resistant clones.

Both expression constructs have been analyzed by restriction enzyme mapping and DNA sequencing.

Sequence analysis of expression constructs

The expression vector constructs have been sequenced and the reported gene sequences compared to the published sequences. Table 2 shows a summary of the amino acid mismatches for the different laminin chains.

$\alpha 3$ chain: the reported sequence matched the published sequence.

$\beta 3$ chain: several discrepancies with the published sequence were found. Single and multiple base deletions and insertions are present along the sequence. These base changes generated some silent mutations, amino acid substitutions and insertion of amino acids. These changes do not cause early termination codons. Therefore, the $\beta 3$ chain seems to be of "full-length" and the protein is being produced.

$\gamma 2$ chain: This chain was reported to have 3 base changes creating 3 amino acid substitutions.

Table 2: Summary of amino acid differences from those reported in the literature

Laminin chain	Amino acid change
α_3	None
β_3	P, insertion at position 251-2 $A_{372} - P_{372}$ $R_{408}R_{409} - Q_{408}G_{409}$ R, insertion at position 421
	$P_{584} - R_{584}$ $A_{796} - G_{796}$ $R_{894}S_{895}E_{896} - S_{894}E_{895}A_{896}$
γ_2	$R_{168} - G_{168}$ $I_{473} - M_{473}$ $S_{521} - N_{521}$

Transfection of human kidney 293 cells

Wild type human kidney 293 cells were transfected with the different expression constructs utilizing standard techniques. Two transfection reagents were used, LIPOFECTAMINE™ from GIBCO (Rockville, MD) and SUPERFECT™ from Qiagen (Valencia, CA). Experiments (see below) suggested that the 293 cells do not express detectable endogenous laminin α_3 , β_3 , or γ_2 chains.

Briefly, both methods required mixing the transfection reagent with the DNA of interest, incubating for a brief period at room temperature, and adding the mixture to the cells. The cells were split the previous day so they were at 50-80% confluence the day of the transfection. The incubation with the DNA-reagent complexes was conducted for 2-3 hours in serum free media for LIPOFECTAMINE™ transfection or complete media for SUPERFECT™ transfection. After this incubation period the media was replaced with fresh growth media and the incubation was continued until the selection process begins.

The selection process was carried out in DMEM F12/10% FBS containing either Geneticin (G418 sulfate) at 400 μ g/ml for selection of G418 resists, or Zeocin at 50 μ g/ml for selection of Zeocin resists. After splitting to selective media, the cells were fed every two days with fresh selective media, until cell foci were identified. Clones transfected with

the three laminin chains and secreting r-laminin 5 into the medium were selected with media containing both antibiotics.

Results

5 Media from human kidney 293 cells transfected with a single laminin chain were initially analyzed on Western blots using chain-specific anti-laminin-5 antibodies. Cell fractions, as well as "whole" fractions containing cells plus any deposited "matrix-like" material obtained by scraping the cells into loading buffer, were also analyzed. Western blot analysis of wild type 293 cell cultures showed no detectable laminin α 3, β 3, or γ 2 chain proteins.
10

The expression of single laminin chains following transfection is generally intracellular, except for a few β 3 clones that appear to show β 3 chain reactivity in the media in Western blot analyses using the anti-FLAG antibody.

15 All clones showing FLAG antibody reactivity were verified by PCR to confirm the incorporation of the transfected gene. Analysis of genomic DNA preparations from such clones by PCR was done using laminin chain-specific primer pairs. The amplified products were compared to positive controls where the original expression constructs were used as templates. Results are shown in Table 3. A few selected clones were analyzed by RT-PCR using the same laminin chain-specific primers and total RNA and/or mRNA preparations as
20 templates. These results are also shown in Table 2.

Other data (not shown) demonstrated that the molecular sizes of some of the components of r-laminin 5 were different from those in purified laminin 5. Particularly, the major component of the α 3 chain in purified laminin 5 was 165 kD, while the α 3 band in r-laminin 5 migrated as two chains of 150 kD and 95 kD.

25 Identified co-transfected clones producing all three chains (as assessed by both genomic PCR and RT-PCR analysis), were further analyzed in a keratinocyte cell adhesion binding assay.

30 *HFK cell adhesion assay for laminin-5.* The method used measures laminin-5 activity present in conditioned media from various clones. Any laminin-5 present in the test media was trapped to a 96 well via an anti-laminin α 3 antibody (C 25). Human foreskin keratinocytes (HFK) were labeled fluorescently, added to the treated wells, and allowed to

adhere for 30 minutes. Fluorescence was measured before and after washing with PBS. The % cell adhesion is equal to fraction of fluorescence retained in the well. As controls, cells were pre-incubated with an anti-integrin $\alpha 3\beta 1$ inhibitory antibody (P1B5) ($\alpha 3\beta 1$ is the cell receptor for laminin 5), or non-specific control antibody (SP2) before being added to the wells. Media controls (Keratinocyte growth media ("KGM"); or DMEM F12 culture media ("medium") were also used. The "a2_F" notation denotes culture medium from 293 cells transfected to express an unrelated FLAG-containing protein.

The results, shown in **Table 2** (last column) and in **Figure 1**. The figure is labeled as follows: C5 and F10: conditioned culture media from r-laminin-5 producing clones C5 and F10; *C6 and *F10: conditioned culture media collected earlier and kept refrigerated. These data demonstrated that media from several clones produced positive results in the cell adhesion assay, indicating the r-laminin-5 produced by these clones is biologically active. The activity was inhibited in the presence of an integrin $\alpha 3\beta 1$ antibody, demonstrating that the r-laminin 5 is binding to the cells via the $\alpha 3\beta 1$ integrin.

To assist in the purification of the heterotrimer r-laminin-5 molecule, the laminin $\beta 3$ chain was labeled with a 'flag' sequence at the amino terminus end. Media from clones transfected with all three chains, and shown to express all three chains, were passed through an anti-flag column and eluted with excess flag peptide. The eluted fractions were analyzed by gel electrophoresis. The data demonstrate that r-laminin 5 was produced and isolated.

20

Table 3: Summary analysis of selected r-L5 clones

Clone	Western Blot				PCR ¹			RT-PCR ²			Adhesion Assay
	$\alpha 3$	$\beta 3$	$\gamma 2$	Flag	$\alpha 3$	$\beta 3$	$\gamma 2$	$\alpha 3$	$\beta 3$	$\gamma 2$	
A2-3	-	nd	nd	+	-	+	+	-	+	+	-
A4-3	+	+	+	+	+	+	+	+	+	+	+
A10-3	-	nd	+	+	-	+	-	-	+	+	-
B1-6	nd	nd	nd	+	+	+	+	+	+	+	+
C2-3	-	nd	+	+	-	+	+	+	+	+	-
C5-7	nd	nd	nd	+	+	+	+	nd	+	+	-
C6-3	+	+	+	+	+	+	+	+	+	+	+
C10-3	-	nd	nd	+	-	+	+	+	+	+	-
E1-3	-	nd	nd	-	-	+	-	-	+	+	-
E2-3	-	nd	+	+	-	+	-	+	+	+	-
E7-3	-	nd	-	+	-	+	-	-	+	+	-
F10-5	nd	nd	nd	+	+	+	+	+	+	+	+

nd = Not determined

1. PCR analysis of genomic DNA preparations were performed using laminin chain-specific primer pairs. The amplified products were compared to positive controls where the original expression constructs were used as templates.

5 2. RT-PCR analyses were done similarly using total RNA and/or mRNA as templates and primers as above.

Several of the above clones were selected for further analysis. A 1 liter culture from
10 clone F10-5 was prepared, and r-laminin 5 was purified using the methods described above. The r-laminin 5 was used in an HFK cell adhesion assay exactly as described above, except that r-laminin 5 was coated directly onto the plate. The results are presented in **Figure 2** and demonstrate that r-laminin 5 markedly increases adhesion of HFK cells at all concentrations tested.

15

Electron Micrograph Analysis

20 Purified r-laminin 5 protein was diluted to 50 µg/ml and adjusted to 70% glycerol/30% 0.15M ammonium bicarbonate and rotary shadowed using standard techniques.

Figure 3 shows an 80,000X magnification field of (A) r-laminin 5; and (B) "native" laminin 5 (purified by BM165 monoclonal antibody affinity chromatography from SCC-25 (squamous cell carcinoma cell line) conditioned medium). The bar represents 50 nm. These results
25 demonstrated that both the r-laminin 5 and the "native" purified laminin 5 formed similar cross-shaped structures typical of laminins.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications
30 may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.